Identification of the Ca^{2+}-Dependent Calmodulin-Binding Region of Chromogranin A

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ABSTRACT: Chromogranin A (CGA), the most abundant protein in bovine adrenal chromaffin granules, is a high-capacity, low-affinity Ca^{2+}-binding protein found in most neuroendocrine cells, and binds calmodulin (CaM) in a Ca^{2+}-dependent manner. The binding of chromogranin A to calmodulin was determined by measuring the intrinsic tryptophan fluorescence of chromogranin A in the presence and absence of Ca^{2+}. Binding was specifically Ca^{2+}-dependent; neither Mg^{2+} nor Mn^{2+} could substitute for Ca^{2+}. Chelation of Ca^{2+} by EGTA completely eliminated the chromogranin A–calmodulin interaction. CaM binding was demonstrated by a synthetic CGA peptide representing residues 40–65. When the CGA peptide and CaM were mixed in the presence of 15 mM $CaCl_2$, the intrinsic tryptophan fluorescence emission underwent a substantial blue-shift, shifting from 350 to 330 nm. Like the intact CGA, the peptide–CaM binding was specifically Ca^{2+}-dependent, and neither Mg^{2+} nor Mn^{2+} could induce the binding. Calmodulin bound both to CGA and to the synthetic CGA peptide with a stoichiometry of one to one. The dissociation constants (K_d) determined by fluorometric titration were 13 nM for the peptide–CaM binding and 17 nM for intact CGA–CaM binding. The K_d values are comparable to those ($\sim 10^{-9}$ M) of other CaM-binding proteins and peptides, demonstrating a tight binding of CaM by CGA. The CaM-binding CGA residues 40–65 are 100% conserved among all the sequenced CGAs in contrast to 50–60% conservation found in the entire sequence, implying essential roles of this region.

The secretory vesicles of adrenal medullary chromaffin cells contain large amounts of catecholamine, ATP, calcium, and an acidic protein, chromogranin A (CGA)[1] (Winkler & Westhead, 1980). Recently, the secretory vesicles were identified as the major inositol 1,4,5-trisphosphate-sensitive intracellular Ca^{2+} store of adrenal medullary chromaffin cells (Yoo & Albanesi, 1990a), and their role as the Ca^{2+} store has been suggested to be due to the high-capacity, low-affinity Ca^{2+}-binding property of chromogranin A (Yoo & Albanesi, 1991). Due to its coexistence with catecholamine, chromogranin A has attracted a great deal of attention since its discovery in the 1960s (Helle, 1967; Smith & Winkler, 1967; Smith & Kirschner, 1967; Blaschko et al., 1967). Nevertheless, elucidation of the physiological functions in the cell remained elusive until recently, partly due to the difficulty of isolating CGA in an intact form. Attempts to purify CGA often ended up with proteolyzed fragments. Frequently, a boiling step was employed to remove other proteins, taking advantage of the heat-stable nature of CGA (Rosa et al., 1985; Rieker et al., 1988). However, this procedure appeared to change the native conformation of CGA as determined by circular dichroism spectroscopy (Yoo & Albanesi, 1990b). During the course of our study of CGA, it was noticed that calcium exposed hydrophobic sites in CGA and this hydrophobicity appeared to contribute to the Ca^{2+}-dependent binding of CGA for calmodulin (Yoo & Albanesi, 1990b). Therefore, the affinity of CGA for CaM in the presence of Ca^{2+} was utilized to purify intact homogeneous CGA from bovine adrenal medulla, and the purified CGA was used to study its interaction with CaM.

Like two other known calcium storage proteins, calsequestrin (MacLennan & Wong, 1971; Ikemoto et al., 1972; Cozens & Reithmeier, 1984) of the sarcoplasmic reticulum (SR) and calreticulin (Ostwald & MacLennan, 1974; Treves et al., 1990) of the endoplasmic reticulum (ER) which have a high content of acidic amino acids (Reithmeier et al., 1987; Fliegel et al., 1987, 1989; Scott et al., 1988; Smith & Koch, 1989) and a high calcium-binding capacity, binding 20–50 mol of Ca^{2+}/mol of protein with a K_d of 1–2 mM (MacLennan & Wong, 1971; Ikemoto et al., 1972; Ostwald & MacLennan, 1974; Cozens & Reithmeier, 1984; Treves et al., 1990), chromogranin A is an acidic protein with $\sim$25% glutamic and aspartic acids (Benedum et al., 1986; Iacangelo et al., 1986; Konecki et al., 1987; Helman et al., 1988), has a p*I* of 4.5–5.0, and binds 32–55 mol of Ca^{2+}/mol of protein with a K_d of 2–4 mM (Yoo & Albanesi, 1991). Chromogranin A also undergoes major Ca^{2+}-dependent conformational changes (Yoo & Albanesi, 1990b) as is the case with calsequestrin (Ostwald et al., 1974). However, unlike calsequestrin or calreticulin which remains in a constant-pH environment, chromogranin A passes through different pH environments during its life cycle in the cell. Beginning from the nonacidic environment of the endoplasmic reticulum (Schwartz et al., 1985; Anderson et al., 1984), the pH environment of CGA becomes increasingly acidic as it moves to the trans-Golgi network (Orci et al., 1986; Anderson & Pathak, 1985) where the secretory vesicles are formed. Finally, in the mature secretory vesicles, CGA is exposed to a pH of 5.5 (Johnson & Scarpa, 1976; Casey et al., 1976) until it is released to the bloodstream where CGA again encounters the physiological pH of 7.2–7.4. We have shown previously that CGA undergoes pH-dependent conformational changes (Yoo & Albanesi, 1990b, 1991), and the pH-dependent conformational changes appeared to contribute to the different

[1] Abbreviations: CGA, chromogranin A; CaM, calmodulin; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; bis-ANS, 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate].

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oligomeric states of CGA (Yoo & Albanesi, 1991). Study of the oligomeric states by analytical ultracentrifugation showed that chromogranin A existed in a dimeric state at pH 7.5 and in a tetrameric state at the intravesicular pH of 5.5 (Yoo & Lewis, 1992), suggesting that CGA goes through a dimeric to a tetrameric state as it moves from the ER and cis-Golgi area to the trans-Golgi network.

Since calmodulin does not have any tryptophan residues of its own, the intrinsic tryptophan fluorescence provides information about the environment of the tryptophan residues of CaM-binding proteins (Malencik et al., 1982; Malencik & Anderson, 1982; Enyedi et al., 1989). Tryptophan is known to fluoresce maximally at ~350 nm in the nonhydrophobic environment, but the emission maximum wavelength decreases as its environment becomes more and more hydrophobic due to its burial in the hydrophobic areas of CaM. Shifts in the fluorescence emission maximum to shorter wavelengths upon binding to CaM have been demonstrated with myosin light chain kinase (Malencik et al., 1982), mastoparan (Malencik & Anderson, 1982), and various peptide hormones (Enyedi et al., 1989). In the present study, binding of CGA to CaM was studied by measuring the intrinsic tryptophan fluorescence. In addition to identify the CaM-binding site of CGA, several synthetic CGA peptides representing various portions of CGA were synthesized, and the CaM-binding ability of each peptide was tested using CaM affinity chromatography and fluorescence spectroscopy. Hence, a CaM-binding segment of CGA was identified, and its CaM-binding parameters were determined using fluorescence spectroscopy.

Experimental Procedures

Materials. $CaCl_2$, $MgCl_2$, Tris, and EGTA were obtained from Sigma, and $MnCl_2$ was from Aldrich Chemical. Cyanogen bromide (CNBr)-activated Sepharose 4B was from Pharmacia LKB.

Preparation of Chromogranin A. Secretory vesicles from bovine adrenals were isolated by a slight modification of the method of Smith and Winkler (1967) as described previously (Yoo & Albanesi, 1990b), and chromogranin A was purified from the soluble vesicle lysates (Yoo & Albanesi, 1990b).

Purification of CaM and Coupling of CaM to Sepharose 4B. Calmodulin was extracted from bovine brain and purified by the method of Dedman and Kaetzl (1983). The purified CaM was free from any contaminants as determined by spectroscopy and was used for CaM titration as well as for coupling to CNBr-activated Sepharose 4B (Yoo & Albanesi, 1990b).

Fluorescence Spectroscopy. Emission spectra were obtained using an SLM 8000C spectrofluorometer. Excitation was at 295 nm. For CGA–CaM-binding studies (Figures 1–3), excitation and emission band-passes were both 4 nm. The fluorescence emission spectra at 320 nm were obtained under various experimental conditions in 15 mM Tris-HCl, pH 7.5, and F/F_0 ratios were plotted as a function of increasing concentrations of either divalent cations or calmodulin. Identical results were obtained when 15 mM sodium acetate, pH 5.5, was used. For peptide–CaM-binding studies, excitation and emission band-passes were set at 4 and 8 nm, respectively, and the peptides were in 20 mM Tris-HCl, pH 7.5, and 0.1 M KCl. All fluorescent measurements were made at 24 °C using a dual-path-length cuvette with the short path length (2 mm) oriented toward the excitation side to minimize inner filter effects.

CGA Peptide Synthesis. Eleven peptides, each representing 20–27 amino acids of various portions of chromogranin A, were synthesized. The synthesized peptides were purified by high-performance liquid chromatography (HPLC), and the integrity of the peptides was ensured through analysis by fast atom bombardment mass spectrometry and by amino acid composition analysis. The purity of all the peptides was 95% or higher. For peptides 1–5, 8, 9, and 11 where there was no tryptophan, a tryptophan residue was added at the N-terminus of each peptide to facilitate analysis.

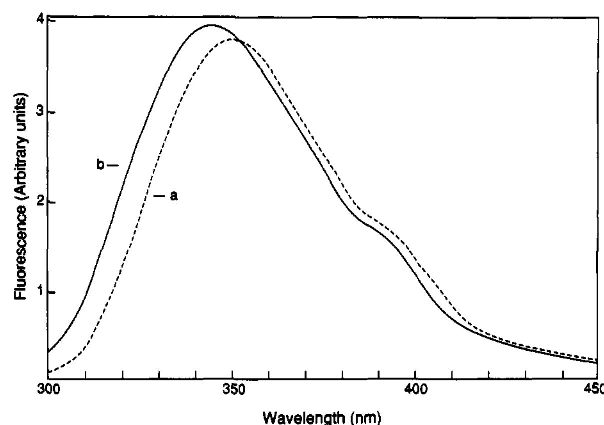

FIGURE 1: Intrinsic tryptophan fluorescence of chromogranin A before and after addition of calmodulin. The intrinsic tryptophan fluorescence was measured in 15 mM sodium acetate, pH 5.5, with 10 μM chromogranin A (a) and 5 μM chromogranin A plus 5 μM calmodulin (b), both in the presence of 15 mM $CaCl_2$. A similar result was also obtained in 15 mM Tris-HCl, pH 7.5.

Calculation of the Peptide–CaM Dissociation Constant. Determination of the dissociation constant was obtained from the fluorescence titration data as described previously (DeGrado et al. 1987; Girsch & Peracchia, 1991). The peptide 2–CaM titration curve was obtained by measuring the fraction of the maximal blue-shift as a function of CaM concentration. The peptide 2–CaM dissociation constant was calculated from the equation (DeGrado et al., 1987; Girsch & Peracchia, 1991):

$$K_d = \frac{(1 - f)([\text{peptide 2}] - f[\text{CaM}])}{f} \qquad (1)$$

where f is the fraction of the maximal blue-shift which occurs at saturating CaM concentrations, and [peptide 2] and [CaM] are peptide 2 and CaM concentrations, respectively. Peptide concentrations were determined spectrophotometrically using 5500 M^{-1} as the extinction coefficient at A_{280}.

Results

To demonstrate the binding of chromogranin A to calmodulin, the effect of CaM on the intrinsic tryptophan fluorescence of CGA was measured. When CGA was excited at 295 nm, it exhibited an intrinsic tryptophan fluorescence emission peak at 350 nm (Figure 1). Addition of CaM not only caused a shift of the fluorescence emission maximum to 344 nm in the presence of Ca^{2+} but also increased the maximal fluorescence intensity. However, these changes were not observed in the absence of Ca^{2+}, indicating Ca^{2+}-dependent CGA binding to CaM.

To determine the effect of various divalent cations on CGA–CaM binding, the changes in the intrinsic tryptophan fluorescence of CGA were monitored in the presence of both CaM and either Ca^{2+}, Mg^{2+}, or Mn^{2+} (Figure 2). Since addition of metal ions (Ca^{2+}, Mg^{2+}, or Mn^{2+}) to CGA alone does not change the intrinsic tryptophan fluorescence of CGA, the change in fluorescence is a direct reflection of the effect of each ion on CGA–CaM binding. As shown in Figure 2A, binding of CGA to CaM was exclusively dependent on Ca^{2+};

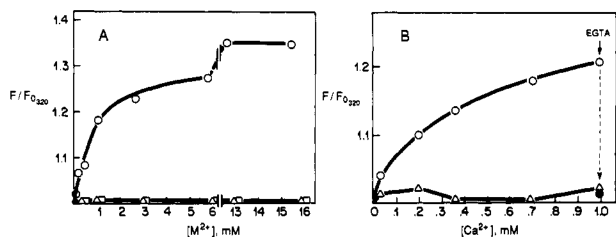

FIGURE 2: Effect of divalent cations on the chromogranin A–calmodulin interaction. The changes in the intrinsic tryptophan fluorescence of CGA were measured by adding Ca^{2+}, Mg^{2+}, and Mn^{2+} to the mixture of CGA and CaM (5 μM each) in 15 mM Tris-HCl, pH 7.5. (A) Fluorescence changes as a function of Ca^{2+} (○), Mg^{2+} (△), or Mn^{2+} (□). (B) Fluorescence changes in the presence (△) and absence (○) of 1 mM EGTA. 1 mM EGTA was added at the end of the titration (●).

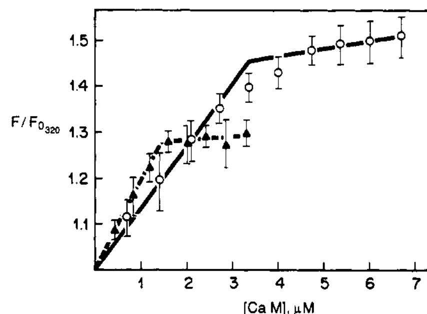

FIGURE 3: Fluorescence titration of chromogranin A with calmodulin. Fixed amounts of CGA [1 μM CGA (△), 3 μM CGA (○)] were titrated in the presence of 15 mM $CaCl_2$ with 220 μM CaM. Data given are mean ± SD of three (▲) and four (○) separate experiments. The ratio of the fluorescence after an increment of CaM (F) over the initial fluorescence (F_0) at 320 nm was plotted as a function of CaM concentration.

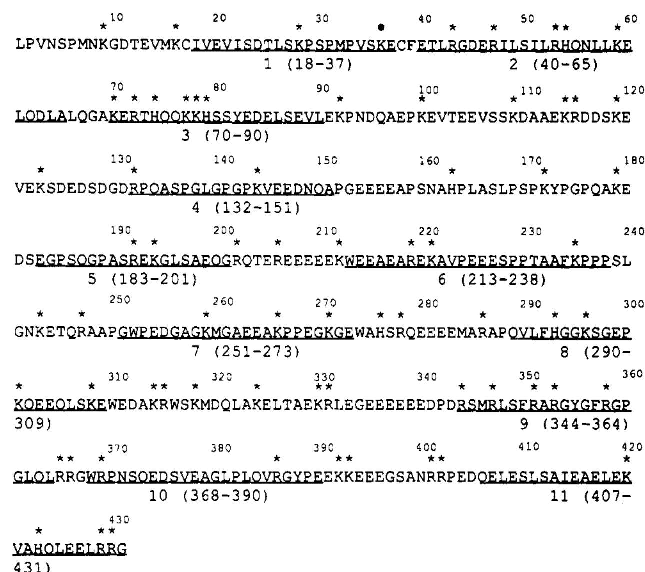

FIGURE 4: Size and location of the synthetic chromogranin A peptides. The amino acid sequence of bovine chromogranin A (Benedum et al. 1986) is expressed in the single-letter code. The peptides were numbered from 1 to 11, and the size of each peptide is indicated by an underline followed by the number of the amino acid location in parentheses. The basic amino acids are denoted with asterisks. For peptides 1–5, 8, 9, and 11, where there was no tryptophan, a tryptophan residue was added at the N-terminus of each peptide to facilitate analysis.

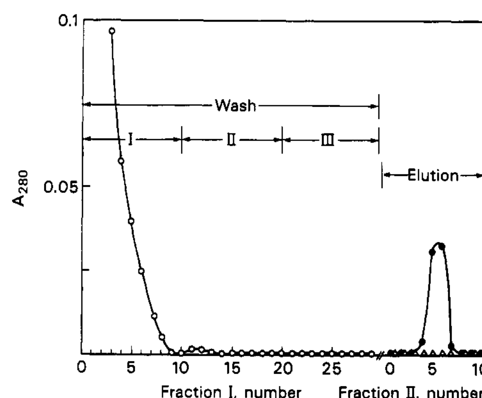

FIGURE 5: Calmodulin–Sepharose 4B chromatography of chromogranin A peptides. One milliliter of each synthetic CGA peptide at a concentration of 1 mg/ml in 20 mM Tris-HCl, pH 7.5, and 0.1 M KCl was loaded onto a CaM–Sepharose 4B column (1.2-mL volume) equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.2 mM $CaCl_2$, and 0.1 M KCl). After the column was loaded, it was thoroughly washed with buffer A and then with buffer A containing 0.5 M KCl. The column was washed a third time with buffer A to reduce the KCl concentration. Chromogranin A peptides which might have been bound to the CaM column were eluted with buffer A containing 1 mM EGTA instead of 0.2 mM $CaCl_2$. The elution profiles of peptide 2 (○, ●) and other peptides (○, △) are shown. Collection volumes are 1 mL/fraction (for fraction I) and 0.5 mL/fraction (for fraction II).

neither Mg^{2+} nor Mn^{2+} could induce the binding. The calcium dependency of the binding reached a steady level at about 12–13 mM Ca^{2+}. To further confirm the Ca^{2+} requirement for the CGA–CaM interaction, the effect of Ca^{2+} on the CGA–CaM interaction was monitored in the presence of EGTA (Figure 2B). In the presence of 1 mM EGTA, addition of Ca^{2+} up to 1 mM failed to induce the CGA–CaM interaction. Further addition of Ca^{2+} in excess of 1 mM caused a gradual increase of the signals. When EGTA was added to the solution containing Ca^{2+}, the signal dropped to the original level, indicating the reversibility of the CGA–CaM interation.

Titration of fixed amounts of CGA with concentrated CaM showed a saturation pattern (Figure 3) which indicated very strong binding. The fluorescence signals increased with increasing CaM concentrations at low levels of CaM. The signals reached a plateau at about 1 μM CaM when 1 μM CGA was used and 3 μM CaM when 3 μM CGA was used, suggesting a one to one binding between CGA and CaM.

To identify the CaM-binding region in chromogranin A, 11 peptides representing various portions of CGA were synthesized (Figure 4). Among them, peptides 2, 3, and 9 contained several basic residues, which is one of the three basic requirements for CaM-binding peptides. These peptides were tested for their Ca^{2+}-dependent CaM-binding ability using CaM affinity chromatography. As the results in Figure 5 showed, peptide 2 (residues 40–65) bound CaM in the presence of calcium and was released from it with the removal of calcium. Of the 11 peptides tested, Ca^{2+}-dependent CaM binding was exerted only by peptide 2. Both peptides 3 (residues 70–90) and 9 (residues 344–364), which contained several basic amino acids among their residues, failed to bind CaM. The helical wheel presentation of peptide 2 shown in Figure 6 shows that the basic amino acids are on one side and the hydrophobic residues on the other although they are dispersed.

To determine whether peptide 2 can have the same effect as intact CGA in binding CaM, the effect of CaM on the intrinsic tryptophan fluorescence of peptide 2 was measured. When the peptide was excited at 295 nm, it exhibited an intrinsic tryptophan fluorescence emission peak at 350 nm (Figure 7). Addition of CaM not only caused the fluorescence emission maximum to be shifted from 350 to 330 in the

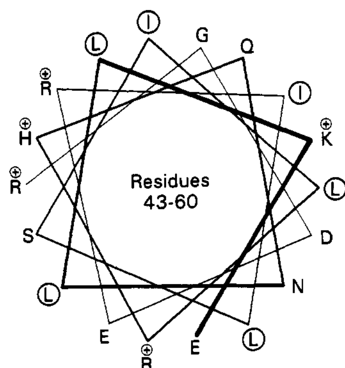

FIGURE 6: Helical wheel representation of chromogranin A peptide 2. Basic residues are denoted with (+), and hydrophobic residues are circled. Residues 43–60 are shown.

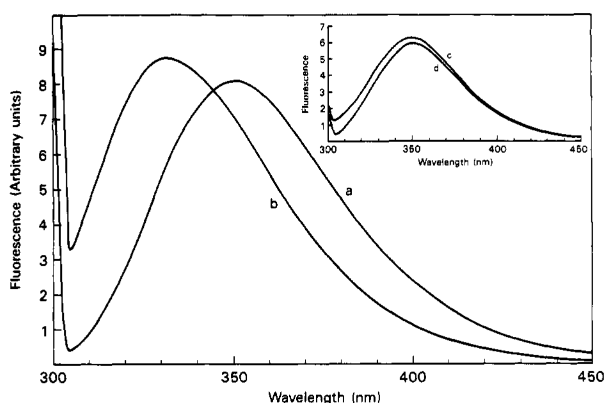

FIGURE 7: Intrinsic tryptophan fluorescence of peptide 2 before and after the addition of calmodulin. Intrinsic tryptophan fluorescence was measured in 20 mM Tris-HCl, pH 7.5, and 0.1 M KCl with 5 μM peptide 2 (a) and 5 μM peptide 2 plus 5 μM CaM (b), both in the presence of 15 mM $CaCl_2$. Inset: Intrinsic tryptophan fluorescence is shown with peptide 3 in the absence (c) and presence (d) of CaM. Other conditions are the same as those with peptide 2.

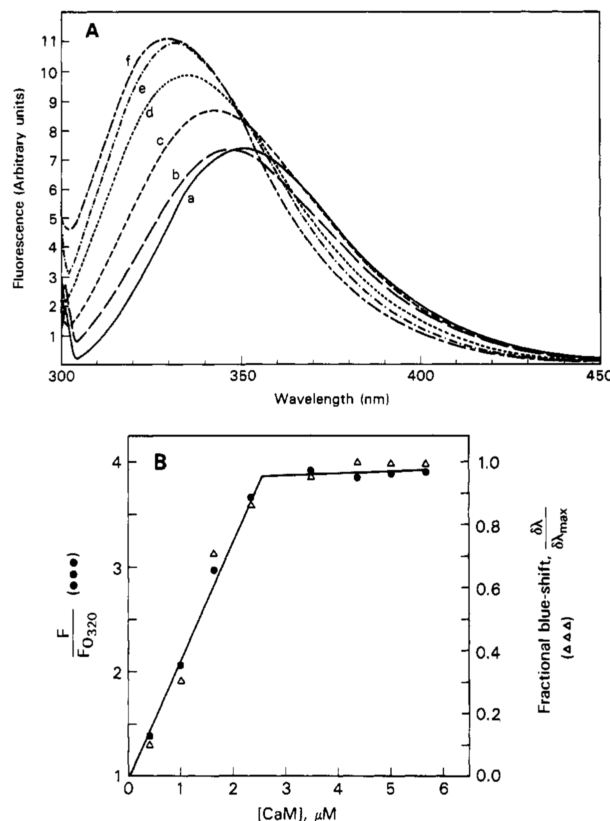

FIGURE 8: Fluorescence titration of peptide 2 with calmodulin. 3 μM peptide 2 in 20 mM Tris-HCl, pH 7.5, and 0.1 M KCl was titrated in the presence of 15 mM $CaCl_2$ with 220 μM CaM. The gradual changes in the intrinsic tryptophan fluorescence as a result of the incremental addition of CaM are shown (A, B). (A) The CaM concentrations are 0 (a), 0.4 (b), 1.0 (c), 1.6 (d), 2.3 (e), and 4.3 μM (f). For clarity, profiles for 3.4, 5.0, and 5.7 μM CaM are not shown. (B) The fraction of the maximal blue-shift [right ordinate (▵)] and the ratio of the fluorescence change (F/F_0) at 320 nm [left ordinate (●)] were plotted as a function of CaM concentration.

presence of Ca^{2+} but also increased the maximal fluorescence intensity substantially. However, these changes were observed neither in the absence of Ca^{2+} nor in the presence of 15 mM Mg^{2+} or Mn^{2+} (not shown), clearly indicating Ca^{2+}-dependent peptide 2 binding to CaM. Other peptides did not exert any effect on the fluorescence signal. One example is shown in the Figure 7 inset where peptide 3, which has several basic residues in its sequence and located next to peptide 2 in intact CGA, failed to change the fluorescence signal.

To determine the affinity of peptide 2–CaM binding, fixed amounts of peptide 2 were titrated with concentrated CaM. As shown in Figure 8, titration of 3 μM peptide 2 with concentrated CaM showed a saturating pattern, indicating very strong binding. The fluorescence signals initially increased with increasing CaM concentrations. The signals then reached a plateau at about 3 μM CaM, suggesting a 1:1 binding between peptide 2 and CaM. Likewise, when 1 μM peptide 2 was titrated with CaM, the fluorescence emission signals reached a plateau at about 1 μM, confirming a 1:1 stoichiometry of binding (not shown). The fraction of the maximal blue-shift in the fluorescence emission signals was plotted as a function of CaM concentration (Figure 8B, right ordinate), and the dissociation constant was determined. Assuming there is one to one binding, the dissociation constants calculated according to eq 1 at the inflection point showed a K_d of 13 nM for peptide 2-CaM binding. The fractional change in the fluorescence emission signals at 320 nm was also plotted as a function of [CaM] (Figure 8B, left ordinate), and the pattern was remarkably similar to that of the fractional change in the maximal fluorescence emission wavelength. This result appeared to suggest that the fractional changes in the fluorescence emission signals at 320 nm can also be used to calculate the dissociation constant. Therefore, the dissociation constant of intact CGA–CaM binding was estimated using the inflection point of 3 μM CGA titration in Figure 3 and eq 1, and a K_d of 17 nM was obtained. These values resemble those ($\sim 10^{-9}$ M) of other CaM-binding proteins and peptides (O'Neil & DeGrado, 1990), and reflect the high-affinity binding of CGA to CaM.

Discussion

We have demonstrated recently that Ca^{2+} binding to CGA exposes previously buried hydrophobic areas in CGA (Yoo & Albanesi, 1990b). This observation was obtained from the results showing that the binding of the fluorescent probe bis-ANS [4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate] to CGA caused a marked enhancement and a blue-shift in the fluorescence emission spectrum. Bis-ANS is the fluorescence probe first described by Rosen and Weber (1969), which exhibits a very low quantum yield in aqueous solvents but a markedly enhanced yield in apolar solvents and upon binding to a number of proteins. Addition of calcium to the CGA–bis-ANS mixture increased the fluorescence emission, suggesting that additional hydrophobic sites on CGA were exposed due to Ca^{2+} binding. This Ca^{2+}-dependent exposure of hydrophobic sites on CGA led us to the possibility of CGA binding to CaM in a Ca^{2+}-dependent manner. Since it has been known that micromolar Ca^{2+} induces conformational

changes of CaM in a manner that exposes hydrophobic sites (Cheung, 1980; Klee & Vanaman, 1982) and millimolar Ca^{2+} can also induce conformational changes of CGA (Yoo & Albanesi, 1990b), the millimolar Ca^{2+} dependency of the CGA–CaM interaction suggests that hydrophobic regions of both proteins are needed for binding.

In the present study, the changes in the intrinsic tryptophan fluorescence signals were used to study Ca^{2+}-dependent CGA–CaM binding. In particular, the shift in the fluorescence emission maximum of peptide 2 from 350 to 330 nm in the presence of CaM and Ca^{2+} (Figure 7) is substantially more pronounced than that seen in intact CGA (Figure 1). Considering that intact CGA contains six tryptophan residues per molecule, the moderate shift of the emission maximum wavelength in intact CGA (6 nm) compared to that in peptide 2 (20 nm) appears to reflect the more averaged environment of the tryptophan residues. The blue-shift in the emission maximum as well as the increase in the signal is suggestive of burial of peptide 2 in the hydrophobic pocket of CaM. These effects were not observed with any other CGA peptides tested. In determination of the dissociation constants (K_d), the expression of a fractional blue-shift in the CGA–CaM interaction as a function of CaM concentration was less informative than a plot expressing the fluorescence increase at 320 nm as a function of CaM concentration due to the narrow range of blue-shift in the CGA–CaM interaction (Figure 1). The K_d of 13 nM for the peptide 2–CaM interaction and of 17 nM for the CGA–CaM interaction generally agree with the dissociation constants determined from many CaM-binding proteins and peptides (Blumenthal & Krebs, 1988; Payne et al., 1988; Kemp et al., 1987; O'Neil & DeGrado, 1990) and demonstrate the high affinity of CGA for CaM in the presence of Ca^{2+}.

Many CaM-binding proteins have been shown to contain a consensus CaM-binding site, i.e., a positively charged, amphiphilic, and α-helical region regardless of its precise amino acid sequence (O'Neil & DeGrado, 1990). A common feature of CaM-binding sites is the presence of several basic amino acids separated from each other by two to three hydrophobic amino acids (O'Neil & DeGrado, 1990). These hydrophobic amino acids provide the α-helical backbone of the CaM-binding region, thereby forming a hydrophobic surface on one side and a positively charged surface on the other. Examination of the primary sequence of bovine CGA to determine whether it contains a possible CaM-binding domain yielded three potential CaM-binding areas: (1) residues 40–65; (2) residues 70–90; and (3) residues 344–364. These three segments appeared to have enough basic amino acids to meet the basic criteria of the CaM-binding sequence. Of these three, peptide 3 did not contain enough hydrophobic residues, making it a less likely CaM-binding segment. Although peptides 2 and 9 have enough hydrophobic residues, the helical wheel presentation of each peptide showed that the hydrophobic residues in peptide 2 are evenly distributed on one side of the helix whereas those in peptide 9 are clustered in two areas. From these analyses, it appeared likely that peptide 2 is the CaM-binding segment. As the results in Figure 5 showed, out of 11 CGA peptides tested, only peptide 2 exhibited CaM-binding ability as had been predicted from the initial analyses.

The lengths of the CaM-binding peptides identified are generally 17–26 amino acid residues long, which is comparable to the length of CGA peptide 2. The helical wheel representation of peptide 2 (Figure 6) shows that the four basic amino acids are aligned on one side while the hydrophobic residues are located on the other. Although the hydrophobic residues look dispersed on the wheel presentation, the division of basic residues and hydrophobic residues appears apparent. Moreover, the secondary structure prediction of this segment also showed mostly α-helical conformation (not shown), which is one of the three common features observed in most of the CaM-binding peptides. Hence, it appears that peptide 2 respresents the CaM-binding region of CGA, satisfying all three requirements generally needed for CaM-binding peptides. The CaM-binding studies carried out with peptide 2 show a stoichiometric binding of CaM to peptide 2 with a K_d of 13 nM. Similar results were also obtained with intact CGA, suggesting that peptide 2 may represent the only area in CGA involved in Ca^{2+}-dependent CaM binding.

	40	50	60
Bovine	ETLRGDERIL	SILRHQNLLK	ELQDLA
Human	----------	----------	------
Pig	----------	----------	------
Rat	---Q----V-	----------	------
Mouse	---Q------	----------	------

FIGURE 9: Comparison of the amino acid sequence of the peptide 2 region of chromogranin A. The amino acid sequences of other known chromogranin As, corresponding to residues 40–65 (peptide 2) of bovine chromogranin A, are compared. Identical residues are shown as dashed lines, and only different residues are shown. Amino acids are expressed in the single-letter code.

Although CGA and CaM are widely distributed in many areas of the brain (Somogyi et al., 1984; O'Connor & Frigon, 1984; Kawakubo et al., 1989; Klee & Vanaman, 1982), calmodulin is not found in the intravesicular milieu. Thus, it is not immediately clear what physiological roles Ca^{2+}-dependent CGA–CaM binding plays in the cell. However, it may be possible for the newly synthesized CGA to come in contact with CaM before its entry into the ER. In light of the fact that the CaM-binding region is near the N-terminus of CGA, it is intriguing to speculate that the N-terminal end of CGA newly emerging from ribosomes may transiently interact with CaM for a variety of reasons, including a proper orientation or an appropriate folding of the N-terminus before its entry into the ER. Interestingly, comparison of all known amino acid sequences of CGA, i.e., human (Konecki et al., 1987; Helman et al., 1988), bovine (Benedum et al., 1986; Iacangelo et al., 1986), pig (Iacangelo et al., 1988a), rat (Iacangelo et al., 1988b), and mouse (Wu et al., 1991), shows a strikingly high sequence conservation of the peptide 2 region (Figure 9). The amino acid sequence in this region is 92.3% identical across the species and 100% conserved, taking the conservative changes into consideration. Although the N-terminal region of CGA, where peptide 2 is located, is one of the two well-conserved regions in CGA, the 92.3% sequence identity of the peptide 2 region shows a sharp contrast to the overall 50–60% homology found in the entire sequence, implying essential roles for this region. Moreover, peptide 2 contains an RGD sequence which is known to participate in the binding of integrin to the extracellular matrix proteins. Although CGA is not an extracellular matrix protein, it is possible that this segment of CGA could be circulating in the bloodstream once CGA and the intravesicular contents are secreted. One interesting possibility is whether this particular segment functions as a yet unidentified peptide hormone or some factor once CGA is proteolyzed after exocytosis. If this is so, it is conceivable that calcium binding or CaM binding to this peptide might serve a regulatory role. In light of the fact that proteolyzed CGA peptides have already been known to function as peptide hormones or regulators of hormone release (Seidah et al., 1987;

Simon et al., 1988; Galindo et al., 1991), it would not be surprising for the CaM-binding segment to exert some form of regulatory function. In addition, peptide 2 also has an ELQD sequence which constitutes the consensus high-affinity calcium-binding sequence in the E-helix region of the calmodulin E-F hand (Cox, 1988; Watterson et al., 1980; Kretsinger, 1980). Given that the ELQD sequence plays a key role in the high-affinity calcium binding of the E-F hand, the presence of an identical sequence in the CaM-binding region of CGA appears to reflect a key role played by calcium in converting the peptide to a conformation conducive to CaM binding.

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Phosphatidylinositol Inhibits Microtubule Assembly by Binding to Microtubule-Associated Protein 2 at a Single, Specific, High-Affinity Site[†]

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ABSTRACT: The effects of various anionic phospholipids on the in vitro assembly of MAP2/tubulin microtubules has been examined. We show that the potency to inhibit is related to the polarity of the phospholipids and that this is consistent with a mode of action involving the sequencing of microtubule-associated proteins (MAPs) by nonspecific electrostatic interactions. The inhibitory potency of phosphatidylinositol (PI) is, however, considerably larger than predicted by this model. The effects of PI on MAP2/tubulin microtubule assembly have therefore been examined in greater detail by preparing phosphatidylcholine (PC) liposomes doped with increasing amounts of PI. We show that when the PI is sufficiently dispersed by dilution with PC, it inhibits microtubule assembly by binding to MAP2 with an apparent stoichiometry, after correction for the bilamellar nature of the liposomes, of 1:1 mol·mol^{-1} PI:MAP2. Furthermore, we show that the K_d of this interaction is in the submicromolar range.

Numerous studies have indicated that microtubule proteins may interact with membrane components, yet the sequences of neither tubulin nor the major neuronal MAPs[1] contain the long hydrophobic peptides characteristic of integral membrane proteins. Tubulin, for example, has been detected in membrane preparations by colchicine binding (Feit & Barondes, 1970; Bhattacharyya & Wolff, 1975), by polyacrylamide gel electrophoresis (Feit et al., 1971; Blitz & Fine, 1974; Kornguth & Sunderland, 1975), by reactivity with specific antibodies (Walters & Matus, 1975), and by tryptic mapping (Kelly & Cotman, 1978), while a particular tubulin isoform specifically associates with membranes in vivo (Hargreaves & Avila, 1985, 1986). In addition, lipids, and particularly phospholipids, are a contaminant of microtubule protein preparations (Daleo et al., 1974; Lagnado & Kirazov, 1975; Hargreaves & McLean, 1988), and the MAPs bind phospholipids tightly (Murthy et al., 1985), while purified microtubule protein causes stacking/fusion of liposomes (Caron & Berlin, 1979) and the enhanced release of material from liposomes (Klausner et al., 1981).

In vitro microtubule assembly is inhibited by membrane components (Daleo et al., 1977; Reaven & Azhar, 1981), an effect which is not nonspecific since membranes from different cellular structures have differing effects (Reaven & Azhar, 1981). Furthermore, pure phospholipids, particularly phosphatidylinositol, inhibit MAP-dependent microtubule assembly in vitro (Yamauchi & Purich, 1987).

We have investigated the inhibition of microtubule assembly in vitro by both pure phospholipids and phospholipid mixtures. We show that phosphatidylinositol inhibits microtubule assembly due to a specific interaction with MAP2 while the activity of other phospholipids is due to nonspecific anionic sequestering of the MAPs.

Materials and Methods

All reagents were obtained from Sigma Chemical Co. Unless otherwise stated, phosphatidylinositol (PI) was the ammonium salt of L-α-phosphatidylinositol extracted from bovine liver, which contains primarily stearic and arachidonic acid. Phosphatidylcholine (PC) was L-α-phosphatidylcholine derived from bovine liver. Phosphatidylglycerol (PG) was the ammonium salt of L-α-phosphatidyl-DL-glycerol prepared by the reaction of cabbage phospholipase D with egg yolk lecithin in the presence of glycerol. Phosphatidylserine (PS) was L-α-phosphatidyl-L-serine prepared from bovine brain. Phosphatidic acid (PA) was either the ammonium salt of dioleoyl-L-α-phosphatidic acid monomethyl ester or the sodium

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[1] Abbreviations: MTP, microtubule protein; MAP, microtubule-associated protein; MES, 4-morpholineethanesulfonic acid; PI, phosphatidylinositol; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-diphosphate; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

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